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Diversispora clara (*Glomeromycetes*)— a new species from saline dunes in the Natural Park Cabo de Gata (Spain)

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Abstract — A new species of *Diversispora* (*Glomeromycetes*) was found in saline sand dunes of the Natural Park Cabo de Gata (Almería, Andalucía, Southern Spain) in the rhizosphere of *Asteriscus maritimus*, a plant species especially adapted to saline environments. The new fungal species forms brilliant white spores that are 79–130 × 75–125 µm and have one wall consisting of three layers. The subtending hyphae are, as typical for many *Diversispora* spp., thin-walled, hyaline, and cylindrical (or rarely constricted) and flexible and fragile below the septa separating the spore and hyphal contents. The septa form regularly at the spore bases or, less frequently, in subtending hyphae at short distances from the spore base. Phylogenetic analyses of the ITS and partial 28S ribosomal gene confirm that *D. clara* forms a monophyletic, independent clade within *Diversispora*.

Key words — *Glomus*, *Glomeromycota*, Europe, extreme environments, rDNA

Introduction

During recent studies on arbuscular mycorrhiza (AM) fungal diversity in sand dune systems of the Cabo de Gata Natural Park in Almería (Spain), a brilliant-white new glomeromycotean fungus was recovered from the rhizosphere of *Asteriscus maritimus*, a plant species characteristic of saline Mediterranean environments with elevated soil electrical conductivity. The new fungus formed spores in AM fungal bait cultures predominantly in the *Asteriscus maritimus* rhizosphere. The aims of the present study were to analyze this particular fungus applying combined morphological and molecular tools and to describe its characteristics.

Material & methods

Soil and plant sampling

Soil samples were taken in February 2010 from the rhizosphere of ten *Asteriscus maritimus* plants growing in a natural sand dune system of the Natural Park Cabo de Gata in Almería (Andalucía, Spain). The site is located at 36°44′41″N 02°07′26″E. The samples, air-dried in the laboratory, were used to analyze selected chemical soil parameters (pH, soil organic carbon, total nitrogen, soil electrical conductivity) and spore populations. The ten plants and the surrounding rhizosphere soil were also extracted and used as bait cultures for propagation of AM fungal communities indigenous to the natural sand dune system. At the site, the soil was sandy and with pH (H_2O) 8.2, organic carbon 15.3 g kg^{-1}, total nitrogen 1.9 g kg^{-1}, available P 27.0 mg kg^{-1}, and soil electrical conductivity 1.5 μS cm^{-1}.

AM fungal bait cultures

Bait cultures were established and maintained as described in Palenzuela et al. (2008, 2011) by transplanting 10 *Asteriscus maritimus* plants with their rhizosphere soils into 1 L pots and transferring them to the greenhouse at EEZ in Granada immediately after sampling. The pots were irrigated three times per week and fertilized every four weeks with Long-Aston nutrient solution (Hewitt 1966). Pure cultures of the new fungus were initiated in a mixed-culture of three *Allium porrum* L. and three *Hieracium pilosella* L. plantlets in 750 mL pots grown together at three locations in the pots. The plant rhizosphere was inoculated with 20 spores per pot and pot location. A sterile mixture of Terragreen (American aluminum oxide, Oil Dry US special, type III R; Lobbe Umwelttechnik Iserlohn, Germany) and Loess (mixture 3:1; with pH-KCl 6.2; organic carbon 0.3%; available P (Na-acetate) 2.6 mg kg^{-1}; available K (Na-acetate) 350 mg kg^{-1} was chosen as culture substrate (Oehl et al. 2002). So far, pure cultures of the new fungus have not been obtained.

Morphological analyses

AM fungal spores were separated from the soil samples by a wet sieving process (Sieverding 1991). The morphological spore characteristics and their subcellular structures were described from a specimen mounted in: polyvinyl alcohol-lactic acid-glycerol (PVLG; Koske and Tessier 1983); a mixture of PVLG and Melzer's reagent (Brundrett et al. 1994); a mixture of lactic acid to water at 1:1; Melzer's reagent; and water (Spain 1990). The spore structure terminology follows Oehl et al. (2003, 2005, 2011a) for species with glomoid or diversisporoid spore formation. Photographs (Figs 1–10) were taken with a Leica DFC 290 digital camera on a Leitz Laborlux S compound microscope using Leica Application Suite Version V 2.5.0 R1 software. Specimens mounted in PVLG and the PVLG+Melzer's mixtures were deposited at the herbaria Z+ZT (ETH Zurich, Switzerland), GDA-GDAC (University of Granada, Spain), and URM (Federal University of Pernambuco, Recife).

Molecular analyses

Five spores isolated from the trap cultures were surface-sterilized with chloramine T (2%) and streptomycin (0.02%) (Mosse 1962) and crushed with a sterile disposable micropestle in 40 μL milli-Q water (Ferrol et al. 2004). PCRs of the crude extracts

were obtained in an automated thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Foster City, California) with a pureTaq Ready-To-Go PCR Bead (Amersham Biosciences Europe GmbH, Germany) following manufacturer's instructions with 0.4 µM concentration of each primer. A two-step PCR amplified the partial SSU, ITS1, 5.8S, ITS2 and partial LSU rDNA ribosomal fragment using the SSUmAf/LSUmAr and SSUmCf/LSUmBr primers consecutively (Krüger et al. 2009). The second PCR products were separated electrophoretically on 1.2% agarose gels stained with Gel Red™ (Biotium Inc., Hayward, CA, U.S.A.) and viewed by UV illumination. The band of the expected size was excised with a scalpel and the amplified DNA was isolated from the gel with the QIAEX II Gel Extraction kit (QIAGEN, Valencia, CA, USA), cloned into the PCR2.1 vector (Invitrogen, Carlsbard, CA, USA), and transformed into one shot TOP10 chemically competent *Escherichia coli* cells. After plasmid isolation from transformed cells, cloned DNA fragments were sequenced with vector primers (White et al. 1990) in both directions by Taq polymerase cycle sequencing on an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Sequence data were compared to gene libraries (EMBL and GenBank) using BLAST (Altschul et al. 1990). The new sequences were deposited in the EMBL database under the accession numbers FR873629–FR873633.

Phylogenetic analyses: The AM rDNA ITS1+5.8S +ITS2 fungal sequences were aligned in ClustalX (Larkin et al. 2007) and edited with BioEdit (Hall 1999) to obtain a final alignment with *Acaulospora laevis* Gerd. & Trappe and *A. lacunosa* J.B. Morton as outgroups. Prior to phylogenetic analysis, the model of nucleotide substitution was estimated using Topali 2.5 (Milne et al. 2004). Bayesian (two runs over 1×10^6 generations with a burn in value of 2500) and maximum likelihood (1000 bootstrap) analyses were performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) and PhyML (Guindon & Gascuel 2003) respectively, launched from Topali 2.5, using the GTR + G model. Neighbor-joining (established with the model cited above) and maximum parsimony analyses were performed using PAUP*4b10 (Swofford 2003) with 1000 bootstrap replications.

Results

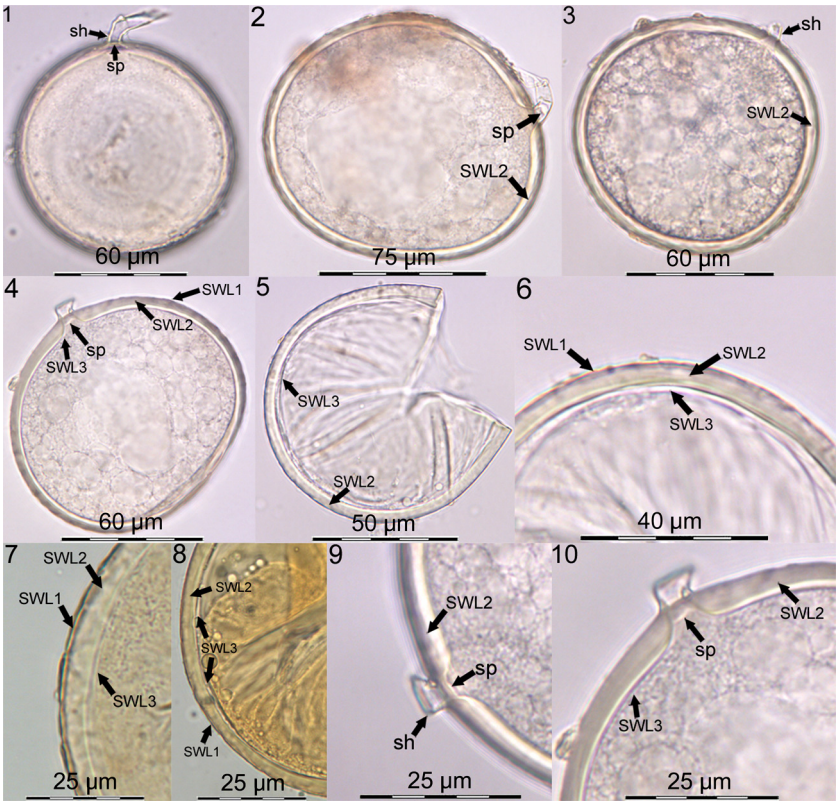
Diversispora clara Oehl, B. Estrada, G.A. Silva & Palenz., **sp. nov.** Figs 1–10

MycoBank MB 561583

Sporae albae, 79–130 × 75–125 µm, tunica tribus stratis, 4.3–8.4 µm. Stratum exterior hyalinum; stratum medium, album ad rarum ochreo-album, 2.8–5.4 µm crassum; stratum interius album. Hypha adhaerenta, 8–13 µm in diametrum. Tunica hyphae 1.0–1.8 µm crassa. Strata medium interiusque septo porum occludentes. Strata exterior mediumque flava colorantes Melzeri.

Type: Spain. Andalucía, Almería, Cabo de Gata Natural Park, sand dune, from the rhizosphere of *Asteriscus maritimus* (L.) Less. (*Asteraceae*), isolation date February 2011, by B. Estrada (**holotype**, ZT Myc 3796 [permanent slide 20-2001]; **isotypes**, ZT Myc 3797 [permanent slides 20-2003 to 20-2012], GDA-GDAC [permanent slides 20-2013 to 20-2018], URM [permanent slides 20-2019, 20-2020]).

Etymology: *clara* (Latin = clear, bright, brilliant, light), referring to the brilliant white spores.



Figs 1–10. *Diversispora clara*: 1–4. Uncrushed spores with a triple-layered spore wall (SWL1-3), a single, cylindrical subtending hypha (sh), and a septum (sp) arising directly or at some distance from the base. Figs 5–6. Spores crushed to show triple layered spore wall (SWL1-3). Figs 7–8. Spore wall structure in PVLG + Melzer's reagent. The SWL1 or SWL2 (or both) stain light to dark yellow in Melzer's, while no such staining reaction is seen on SWL3. However, the yellow stain is usually most noticeable in the spore cell contents. Fig. 9. Septum at spore base formed by SWL2 (SWL3 is not visible in uncrushed spore). Fig. 10. Septum at spore base formed by SWL3.

Spores (Figs 1–4) singly formed in rhizosphere soils, terminally on subtending hyphae, globose (80–110 µm diam.) to subglobose (79–130 × 75–125 µm), one-walled, brilliant to creamy white.

Spore wall 4.3–8.4 µm thick, three-layered (SWL1, SWL2, SWL3; Figs 5–7); outer layer (SWL1) hyaline, 0.8–1.5 µm thick, evanescent and thus often absent in mature spores; second layer (SWL2) bright to (rarely) creamy white, laminated, 2.8–5.4 µm thick (Fig. 7) in uncrushed spores (sometimes ≤ 6.6 µm in crushed spores when pressure is applied on the cover slip); inner layer (SWL3)

brilliant white, 0.7–1.5 µm thick (usually tightly adherent to SWL2 and difficult to observe when < 1.0 µm; see FIGS 5–6); both SWL1 and SWL2 generally light to dark yellow in Melzer's reagent (FIGS 7–8, with spore cell contents often a more intense yellow than the surrounding cell wall layers (FIG. 8).

SUBTENDING HYPHAE (sh) generally singly on spores; brilliant white (FIGS 1–3), cylindrical or (rarely) slightly constricted at the spore base, 4.0–8.0 µm broad tapering to 3.2–6.1 µm within 100 µm of spore base, although the distance may appear shorter (4.0–10(–25) µm) because the sh walls taper from 1.0–1.8 µm thick to 0.6–1.2 µm within the first 10 µm from the base causing the flexible fragile portion to break from the mature spore (FIGS 3, 4, 9) at a point where the septum separates the spore contents from the hyphal contents. Spore pores at the spore bases or in the sh normally closed by a septum (FIGS 1–2, 4) arising from SWL2 (FIG. 9), SWL3 (FIG. 10), or both layers (not shown); pores rarely open (FIG. 3).

DISTRIBUTION: Known only in the natural sand dune system of the Cabo de Gata Natural Park in Andalucía in the rhizosphere of *Asteriscus maritimus*.

MOLECULAR ANALYSES: Phylogenies derived from ITS (FIG. 11) and 28S (data not shown) rDNA analyses cluster the new fungus within the *Diversisporaceae* in a well-separated clade adjacent to several other *Diversispora* species, *Otospora bareae,* and *Tricispora nevadensis* (Oehl et al. 2011b).

Discussion

Our morphological analyses, in particular those of the subtending hyphae and spore bases, clearly support the new fungus in *Diversispora*, with many characters identical to those of other *Diversispora* species (e.g., *D. spurca, D. eburnea;* see Oehl et al. 2011a,b) such as the thin-walled, hyaline, cylindrical (or rarely constricted) subtending hyphae that appear flexible and fragile behind the septum that closes the pore at the spore base or in short distance from the base. In *Diversispora* species with pigmented spores, the subtending hyphae regularly change color conspicuously, becoming hyaline to white behind the septum (Oehl et al. 2011a). This color change, however, was not confirmed for *D. clara*, since the new fungus generally does not form pigmented spores.

Molecular analyses confirm the species as new: in the phylogenetic tree, *D. clara* clusters in a independent, monophyletic clade within a polyphyletic *Diversispora*. These sequence analyses also confirm unequivocally using morphology to identify diversisporoid species within the *Glomeromycota* (Oehl et al. 2011a,c).

Including *D. clara*, there are now 14 *Diversispora* spp. known in the *Glomeromycetes* (Oehl et al. 2011a,b). The 9 species that form significantly pigmented, yellow brown to brown or orange to orange brown spores are *D. arenaria* (Błaszk. et al.) Oehl et al., *D. aurantia* (Błaszk. et al.) C. Walker & A. Schüssler, *D. epigaea* (B.A. Daniels & Trappe) C. Walker & A. Schüssler,

D. insculpta (Błaszk.) Oehl et al., *D. przelewicensis* (Błaszk.) Oehl et al., *D. pustulata* (Koske et al.) Oehl et al., *D. tenera* (P.A. Tandy) Oehl et al., *D. trimurales* (Koske & Halvorson) C. Walker & A. Schüssler, and *D. versiformis* (P. Karst.) Oehl et al. (Oehl et al. 2011a,b). *Diversispora celata* C. Walker et al. forms triple-layered, ochre to ivory to pinkish cream spores (Gamper et al. 2009). Only *D. spurca* (C.M. Pfeiff. et al.) C. Walker & A. Schüssler, *D. eburnea* (L.J. Kenn. et al.) C. Walker & A. Schüssler, and *D. gibbosa* (Błaszk.) Błaszk. & Kovács form hyaline to subhyaline spores that are, however, never brilliant-white as observed for *D. clara*. Moreover, *D. spurca* and *D. eburnea* have bi-layered spore walls (Kennedy et al. 1999), *D. gibbosa* has a five-layered wall (Błaszkowski 1997), and *D. clara* has a three-layered wall.

In the past, large-spored or sporocarpic AM fungi were described from sand dune systems, such as *Gigaspora*, *Scutellospora*, *Pacispora* or *Glomus* species that were generally easy to isolate and recognize from field samples (Koske & Gemma 1995, Gemma et al. 1989, Błaszkowski 1994). Likewise in the current Cabo de Gata Natural Park sand dune system, where *Funneliformis coronatus* (Giovann.) C. Walker & A. Schüssler, *F. mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüssler, *Scutellospora calospora* (T.H. Nicolson & Gerd.) C. Walker & F.E. Sanders, *Racocetra persica* (Koske & C. Walker) Oehl et al., and *Glomus macrocarpum* Tul & C. Tul. were identified (Estrada, unpublished). When sand dune AM fungal communities were maintained and reproduced in bait cultures, small-spored *Glomus* spp. were also sometimes detected (e.g. Błaszkowski et al. 2009a,b, 2010). It was supposed that species with small, quickly degrading spores were difficult to recover or identify only from field samples. This might also be true for *D. clara*, even though its laminated wall structure is clearly persistent. It will be interesting to see whether future taxonomists will be able to identify the new species directly from field samples, now that the existence and morphology of this unique, brilliant-white, conspicuous but small-spored species is known. Morphological spore and molecular root and spore analyses will hopefully tell us more about the ecology and biogeography of this fungus that is thus far known only from a single *Asteriscus maritimus* rhizosphere in the Natural Park Cabo de Gata of Almería in southern Spain.

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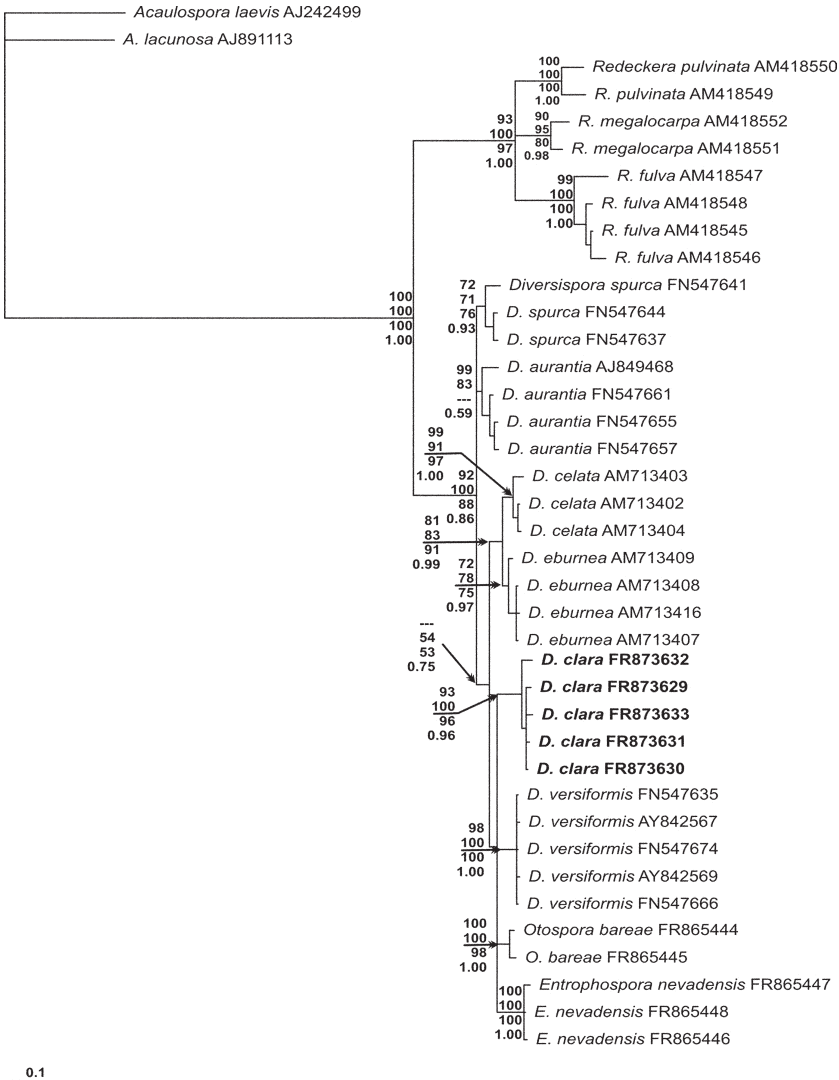

Fig. 11. *Diversisporaceae*. ITS rDNA-based phylogenetic tree rooted by *Acaulospora laevis* and *A. lacunosa*. Sequences are labeled with database accession numbers. Support values are from neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and bayesian analyses. *Diversispora clara* sequences are in bold. Only topologies with ≥ 50% bootstrap values are shown. (Consistency Index = 0.72; Retention Index = 0.84).

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